ON PIG HEART ACONITASE

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Summary. Pig heart aconitase has been isolated by sequential application of ethanol fractionation, carboxymethylcellulose ion-exchange, ammonium sulfate fractionation and isoelectric focusing. The protein, pI 8.5, homogeneous on gel electrophoresis, gel filtration and ultra-centrifugation, manifests a molecular weight of 66,000 (gel filtration, Sephadex G-200) and contains 2 g-atoms of iron and 3 moles of sulfide per 66,000 g.

We report herein the isolation of aconitase from pig heart by a procedure differing in detail from that recently reported (1), our procedure involving stabilization of the enzyme by tricarballylate (2) and the application of isoelectric focusing for final purification, initial purification being achieved by ethanol fractionation, carboxymethylcellulose ion-exchange and ammonium sulfate fractionation, in that order. Details of the procedure are given in Table I, steps 1 through 4 being modifications of existing procedures (1,2,3) and step 5, isoelectric focusing (4), being carried out over a narrow pH range by utilization of a pH 7 to 9 ampholine mixture.

The results of a typical isoelectric focusing run are presented in fig. 1. Enzyme is found in one major peak, pI 8.5, and in two minor peaks, pI 8.1 and 7.9, the second minor peak being insignificant. The pI 8.5 peak and the pI 8.1 peak give aconitase of the same iron content and the same molecular weight (gel-filtration) and, presumably, these peaks represent aconitase isoenzymes. In this connection, the enzyme isolated by

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TABLE I

ISOLATION SEQUENCE[a]

Step	ml	mg/ml[b]	Spec. Act.[c]	Tot. Act.	%
1. Extraction, 1 kg frozen pig heart[d]	3000	5.0	0.38	5700	100
2. EtOH fractionation, 20-40%[e]	500[f]	9.5	0.88	4180	73
3. Ion-exchange[g]	150	8.0	1.56	1870	33
4. $(NH_4)_2SO4$ fractionation[h]	12.5	50	1.85	1160	21
5. Isoelectric focusing[i], main peak	30	2.33[j]	10.2	715	12.5

a. All steps at 0-4° unless otherwise noted.
b. Protein by trichloroacetic acid precipitation, serum albumin standard.
c. Units per mg protein; one unit equal to 1 µmole of TPNH per min; assay according to Rose and O'Connell, ref. 16; all assays carried out after activation with ferrous ion and cysteine.
d. 3 min. blending with 400 ml of $CHCl_3$ and 3 l of 4mM K^+ tricarballylate - 10 mM mercaptoethanol-0.05 mM EDTA.
e. -7 to -12°.
f. Precipitate dissolved in 2mM tris-tricarballylate - 0.15 mM EDTA, pH 6.1.
g. Carboxymethylcellulose, Whatman Cm-52, 12 X 2.5 cm column, linear gradient - elution with 2mM tris-tricarballylate - 0.15 mM EDTA, pH 6.1, and 30mM tris-tricarballylate - 0.15 mM EDTA, pH 6.1.
h. From 0.55 to 0.70 saturation, precipitate dissolved in 15 mM tris-tricarballylate, pH 7.8, and dialyzed against the same buffer.
i. LKB, Model 8102, 440 ml column, sucrose gradient, ampholines pH 7-9, 96 hrs., 600-750 volts.
j. From absorbance at 280 nm, $A^{1\%} = 11.5$.

Villafranca and Mildvan (1) demonstrates on cellulose acetate electrophoresis a pI in the range 9.0 - 9.3. However its iron content, 1 g atom per 89,000 g, differs considerably from the pI 8.5 and pI 8.1 enzymes and the question of an isoenzyme of pI 9.0 - 9.3 remains to be answered. It may also be noted that Eanes and Kun (5) have reported the presence by isoelectric focusing of more acidic aconitases in unfractionated extracts and that we have found (6) by gel isoelectric focusing and by column isoelectric focusing, aconitases* over a wide pH range in unfractionated extracts.

On gel-filtration by the method of Andrews (9) with 50 nM tris-chloride - 0.1 potassium chloride, pH 7.5, buffer and applying 2 mg of enzyme to a

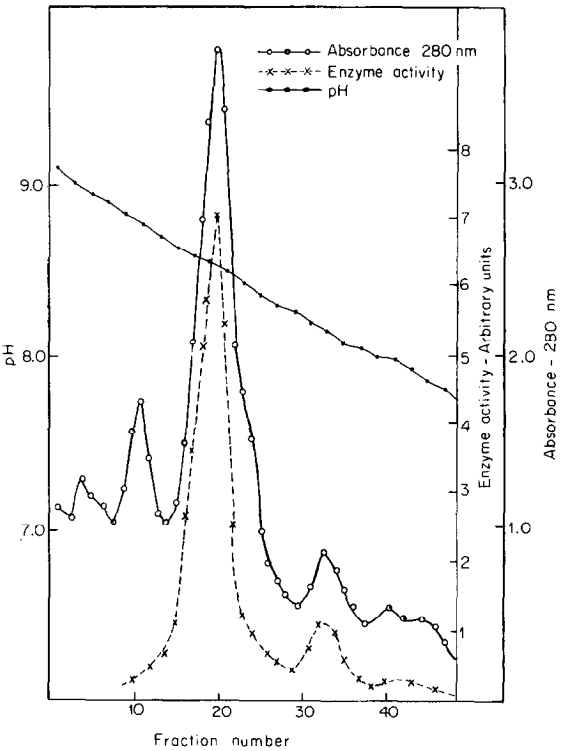

Fig. 1. Isoelectric focusing as described in Table 1, footnote i, 3 ml fractions collected by displacement with water.

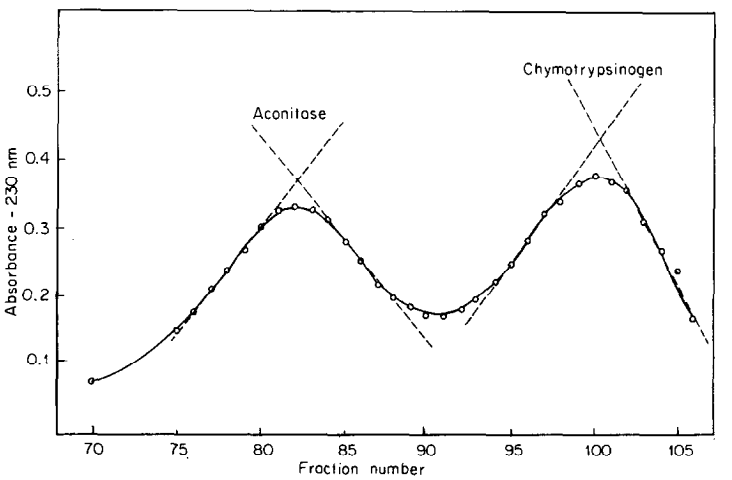

Fig. 2. Gel-filtration of aconitase, pI 8.5. Conditions given in the text.

2.5 x 75 column of G-200 aconitase emerges (fig. 2) as a symmetrical peak (66,000 ± 3,000) in a position immediately following that of bovine serum albumin (mol wt. 67,000) in the calibration procedure.

Elution with tris-citrate, potassium chloride or tris-tricarballylate, potassium chloride, both at pH 7.5, also gives a molecular weight of 66,000 while elution after reductive carboxymethylation and after maleylation gives evidence of association as does elution at pH 4.0 with acetate buffer. Preliminary ultracentrifugation studies give molecular weights that vary with the conditions of the experiment, evidence for both association and dissociation of the 66,000 molecular weight unit being obtained.* Peptide mapping (1) of tryptic digests (10) of aconitase reduced and carboxymethylated according to the procedure (11) employed for ferredoxin, yields ∿35 trypsin peptides and this result suggests, on the basis of a combined lysine and arginine content of ∿70 residues per 66,000 g, that the aconitase molecule contains two identical or closely related sub-units.

Iron determinations by atomic absorption and by spectrophotometry with o-phenanthroline after trichloracetic acid extraction (12) indicate 2 g atoms of iron per 66,000 g. Sulfide determinations by the Bumbry et al (13) modification of the method of Fogo and Popowsky (14) give 3 moles of sulfide per mole of protein.

Preliminary ESR data indicate Fe^{III} as the oxidation state of the iron, a "g" value of 4.25 being found. A comparable "g" value, 4.1, has been found (1) for aconitase isolated by Villafranca and Mildvan (1) and in this respect the iron is in the same state in both proteins. Further, the absorption spectrum (fig. 3) of our preparation shows the same sharp 280 nm maximum and the same flat 320 nm to 600 nm absorption as the Villafranca and

*Demonstrated in the gel by the staining technique of Koen and Goodman, ref. 7.

*The Villafranca and Mildvan preparation shows the same type of ultracentrifugation behavior as does our preparation, personal communication, Dr. J. Villafranca.

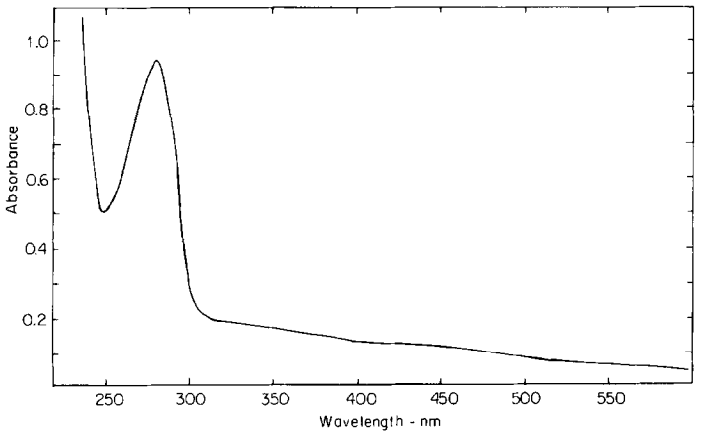

Fig. 3. Absorption spectrum of aconitase, 0.81 mg per ml, 0.015 M tris-tricarballylate, pH 7.8, 1 cm cuvette, 23°.

Mildvan preparation albeit the molar absorbance at 320 nm for our preparation is greater than that found for the latter preparation. We also find ferrous iron activation kinetics and specific activity after activation to be comparable to that found by Villafranca and Mildvan (1). It may, therefore, be concluded that both proteins are identical except for iron content and that activation with ferrous ion and reductant either adds iron to the Villafranca-Mildvan protein or removes iron from our preparation. Since the iron content of our preparation does not change on activation (15), the former possibility is more likely.* Aconitase, as isolated, is then an iron-sulfur protein, possibly of the non-heme iron type, and its characterization as well as its interaction with other components of the Krebs' cycle and with components of the respiratory chain are under investigation.

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*The sulfide content of the Villafranca-Mildvan preparation has, as yet, not been investigated.

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